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(54) Title: IMMORTALIZED HUMAN HEPATIC CELL LINE

(57) Abstract

An immortalized human hepatic cell line is disclosed. The cell line is created by lipofection of primary cultures of normal human fetal hepatic cells with a plasmid containing the second and third exons of the human *c-myc* gene driven by an SV-40 promoter. Cytochemical, morphological and phenotypical analyses indicate that the resulting cell line has hepatocyte characteristics. When these cells were analyzed after 90 passages they continued to express selected hepatocyte markers, including albumin and gamma-fibrinogen. Transmission electron microscopy reveals that the cells exhibit epithelial features, including desmosomal junctions and numerous surface microvilli, and form structures that resemble bile canaliculi. Methods of making the disclosed immortal hepatic cell line are also disclosed.

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IMMORTALIZED HUMAN HEPATIC CELL LINE

This invention was made with United States Government support under grants CA 42765 and CA 56950 from the National Institutes of Health. The United States Government has certain rights to this invention.

Field of the Invention

This application relates to immortalized cell lines, and more specifically to immortalized, human hepatic cells.

Background of the Invention

Isolated hepatocytes in primary culture have been used to investigate gene expression regulation, and both endobiotic and xenobiotic metabolism. These investigations have been limited by the rapid phenotypic changes that occur in dissociated hepatocytes under conventional cell culture conditions, which changes lead to a significant decrease or the complete disappearance of highly differentiated cell functions (*e.g.*, the loss of cytochrome P450 activity and reduced production of plasma proteins). Human hepatocyte cell lines derived from human hepatocellular cancers have been propagated *in vitro* and some exhibit selected characteristics of differentiated human hepatocytes. The derivation of these cell lines from cancers and the observation of abnormal morphology and gene regulation in these lines has raised interest in the development of cell cultures derived from normal human livers. Recently, hepatic epithelial cells capable of propagating in culture have been established from normal human livers by the introduction of SV40 virus genes (e.g., the T-antigen gene), and immortalized cell lines have been developed from them. A.M.A. Pfeiffer et al., *Proc. Natl. Acad. Sci. USA* **90**, 5123-5127 (1993). These cells have been shown to exhibit some of the metabolic characteristics

expected of hepatocytes, including the capacity to metabolize some carcinogens. However, these cells have an undifferentiated appearance that is often seen in cultured human cells infected or transfected with SV40. Moreover, the T-antigen has been shown to associate with tumor suppressor genes. *See* DeCaprio et al., *Cell* **54**, 275-83 (1988). This association may add undesirable changes to the cellular characteristics including karyotype instability, de-differentiation, and transformation over repeated passaging.

A number of studies have shown that a high level of expression of the *c-myc* gene characterizes normal human liver development and regeneration, and is often found in hepatocellular cancers. Past studies have also suggested the importance of detecting the expression of the *c-myc* gene product in normal human hepatic development, differentiation and neoplasia. *See e.g.*, T. Iwanaga et al., *Biomed. Res.* **7**, 161-66 (1986); N. Voravud, et al., *J. Hum. Pathol.* **12**, 1163-1168 (1989). In 1983, it was shown that primary rat embryo fibroblasts have a higher frequency of infinite life span cells after transfection with the *c-myc* oncogene. H. Land, et al., *Nature* **340**, 596-602 (1983). More recently, others have found that the most efficient immortalization construct in mouse cells is a truncated *c-myc* gene driven by an SV40 promoter. J.M. Adams et al., *Nature* **318**, 533-538 (1985).

In spite of these developments, there remains a need for the establishment of an immortalized human hepatic cell line that has the characteristics of normal hepatocytes.

Summary of the Invention

Described herein is the establishment of an immortal, human hepatic cell line derived from normal human fetal liver. This cell line is created by the transfection of normal fetal liver cells with a truncated human *c-myc* construct driven by an SV40

promoter, and the subsequent selection of propagable cells with the integrated gene. This cell line, referred to herein as JB-HEP1, is immortal, yet preserves morphological features of differentiated hepatocytes and retains major regulatory pathways that control hepatocyte gene expression and differentiation.

Accordingly, one aspect of the present invention is an immortalized human hepatic cell line. This immortal cell line is characterized by an exogenous gene integrated into the cellular genome; expression of at least one human liver secretory protein; and morphology consistent with normal hepatocytes. In a preferred embodiment, the cell line is derived from normal fetal liver cells.

The foregoing and other aspects of the present invention are explained in detail in the specification and claims set forth below.

Brief Description of the Drawings

FIG. 1A is a light micrograph showing the morphology of a cell taken from the JB-HEP1 cell line in monolayer culture, and illustrating the hepatocyte cord-like structure in non-confluent cells.

FIG. 1B is a light micrograph showing the morphology of the JB-HEP1 cell line in monolayer, confluent culture.

FIG. 1C is an oil-immersion light micrograph showing detailed hepatocyte structure of the JB-HEP1 cell line in culture.

FIG. 2 is a karyotype of a cell of the JB-HEP1 cell line at the 90th passage. The chromosome complement is near tetraploid with several derivative chromosomes.

FIG. 3A is a transmission electron micrograph of the JB-HEP1 cell line at the 90th passage, illustrating the ultrastructure of the cells in monolayer culture.

FIG. 3B is a transmission electron micrograph of the JB-HEP1 cell line at the 90th passage,

illustrating glycogen storage by dexamethasone treatment.

FIG. 3C is a transmission electron micrograph of the JB-HEP1 cell line at the 90th passage, illustrating the bile canaliculi ultrastructure.

FIG. 3D is a transmission electron micrograph of the JB-HEP1 cell line at the 90th passage, illustrating the D-clathrin lined pits at the basal side of the cells in monolayer culture.

FIG. 4 is a Southern Blot analysis of cellular DNA from the *c-myc* transfected JB-HEP1 cell line, and normal fetal liver. Genomic DNA was extracted from the JB-HEP1 cells line and normal fetal liver. 10μg of each DNA sample were digested with *Eco*R1, which has a single site in the vector DNA (lanes 1 and 5). The DNA samples were also digested with *Bgl*I (lanes 2 and 6), *Eco*RI and *Bgl*I (lanes 3 and 7), and *Pvu*II (lanes 4 and 8). 50, 100, 200, and 500 picograms of plasmid DNA were linearized with EcoR1 as a positive control. Hybridization was done with linearized plasmid. The integration of the 6.5 kB linearized plasmid with *Eco*R1 into the genome of the JB-HEP1 cell line and the 6.5 kB linearized plasmid are indicated. The molecular weight markers are *Hind*III fragments of lambda DNA.

FIG. 5 is a Northern Blot analysis of 5μg of poly(A)$^+$ RNA from the JBH1, JB-HEP1, JBH2 and normal fetal liver, hybridized to a linearized 6.5 kB plasmid. A 2.4 kB normal *c-myc* transcript is expressed in the immortalized JB-HEP1 cell line and the control cells from normal fetal human liver.

FIG. 6 shows three autoradiograms of blots illustrating the expression of albumin, α-fetoprotein and gamma-fibrinogen in the JB-HEP1 cell line.

Detailed Description of the Invention

As summarized above, this invention relates to the development of an immortal cell line of human

hepatic cells derived from normal human fetal liver. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods or materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications and patent documents referenced in this application are incorporated herein by reference.

A. PRODUCTION OF CELLS OF THE INVENTION.

Human hepatic (liver) tissue useful in the present invention may be obtained as surgical excess tissue from aborted human fetuses at 10 to 24 weeks gestation, preferably at 16 to 20 weeks gestation and most preferably at about 18 weeks gestation. Liver tissue is separated from other fetal tissue by any method known to those skilled in the art, and is preferably taken from the right lobe of the fetal liver. The isolated tissue, which will preferably consist mostly of parenchymal hepatic cells, may be minced into smaller pieces and passed through sterile gauze or the like to further separate the cells. Optionally, the cells may be washed with an antibiotic solution, the selection of which antibiotics will be within the skill of the ordinary artisan, and may be selected from the group consisting of penicillin, kanamycin and amphotericin. After washing, the fetal liver cells may be resuspended in a suitable, serum-free medium. As used herein, the hepatic cells of the present invention are defined as cells that are liver cells or are derived from liver cells, with hepatocytes being preferred.

Growth media useful in the present invention is preferably a conditioned growth medium containing hepatocyte growth media (HGM) and MITO+. MITO+ is commercially available from Collaborative Biomedical Products, in Bedford, Massachusetts, USA. The media may

be conditioned by HepG2 cells, and may additionally contain other components, including but not limited to Ham's-F12/M-199 (1:1) supplemented with MITO+, ITS (6 μg/mL insulin, 6 μg transferrin and 6 ng/mL selenium), bovine pituitary extract, insulin and gentamicin sulfate.

Cells of the present invention are immortalized by the transfection of an exogenous, cell growth-potentiating gene (i.e., an oncogene) into the cell. As used herein, an exogenous gene is a nucleic acid sequence not naturally found in the genome of the cell into which the gene is being transfected. The exogenous gene may comprise an entire gene or coding sequence, or some fragment or fragments thereof. In a preferred embodiment of the invention, the transfected gene is the human *c-myc* gene. In a most preferred embodiment, the cells are transfected with a plasmid, and the plasmid is a construct known as pRHP1 (*see* Example 3, below). The pRHP1 construct is a 6.5 kilobase recombinant plasmid containing a portion of the human *c-myc* gene (exon 2 and exon 3), is driven by an upstream SV-40 promoter, with both gene fragment and promoter being integrated into a parental pBR322 plasmid. The genes useful in the present invention may be transfected into the hepatic cell by any method known to one skilled in the art, but are preferably transferred into the genome by lipofection.

In the cell line of the present invention, the hepatic cell lines are grown in serum-free or chemically defined medium to minimize the loss of differentiated cell functions.

B. CHARACTERIZATION OF CELLS OF THE INVENTION

The JP-HEP1 cell line comprises cells containing an intact, truncated human *c-myc* gene driven by an SV40 promoter, as may be confirmed by polymerase chain reaction (PCR) or Southern blot analysis of genomic DNA from the cells. The fidelity of the transfected gene may additionally be confirmed by

Northern blot analysis, which indicates the expression of a single, full-length 2.4kB normal *c-myc* transcript. The JB-HEP1 cell line maintains a continuously increased level of *c-myc* expression (Poly (A)· RNA) of up to about five times the amount expressed by a control specimen (e.g., liver cells of an 18-week, normal human fetus). In the present invention, the enforced expression of *c-myc* prevents human fetal hepatocytes from entering the terminal differentiated pathway, thus keeping them in a continuous proliferative state. However, immortalization with the *c-myc* construct does not prevent the maintenance of a well-differentiated phenotype.

The cells of the present invention are of hepatic origin and maintain several critical features of differentiated hepatocytes. Morphological studies reveal the cells to have cytoplasmic organelles and nuclear structure that are more complex than most cultured epithelial cells. Additionally, they possess an ultrastructure similar to the ultrastructure of hepatocytes *in vivo*. The cells have desmosomes and the cytokeratin complement of secretory epithelial cells. Although the cells express cytokeratins 19 at the 90th passage, they are negative for both keratins 7 and 19 at earlier passages, indicating that they are not like differentiated bile duct epithelial cells. Most importantly, these cells contain glycogen and appear to form structures like bile canaliculi with numerous microvilli at sites of contact between adjacent cells.

The cells of the present invention have a subtetraploid karyotype at the 90th passage with relatively few gross chromosomal abnormalities. They express the mRNA for the albumin gene and in early passages express the alpha-fetoprotein gene. When treated with dexamethasone, these cells accumulate large quantities of glycogen, and induce tyrosine amino transferase activity, as has been seen in rodent and human hepatocytes *in vitro*. *See e.g.*, N. Ruiz Bravo et

al., *Proc. Natl. Acad. Sci. USA* 79, 365-368 (1982); M. Nagao et al., *J. Exp. Med.* **152**, 23-29 (1987). Together, these features indicate that these immortal human fetal hepatic cell cultures maintain differentiated morphological and functional features of hepatocytes.

C. APPLICATIONS OF CELLS OF THE INVENTION

In general, the immortal hepatic cell line of the present invention is useful as an *in vitro* model for replication of human fetal hepatocytes, for the study of genetic regulatory pathways that control hepatocyte differentiation, and for the study of endobiotic and xenobiotic metabolism and the acute phase reaction. The indefinite life span of the human hepatic cell cultures provided by the present invention also makes the cells useful as a more reproducible *in vitro* model for the investigation of many facets of human liver function and the development of liver diseases. For example, the cell line may be used as a model for the study of hepatotoxicity and hepatocarcinogenicity, the production of plasma proteins (e.g., albumin) and their expression, the isolation of hepatocyte-specific genes and transcription factors, the effects of hormonal and inflammatory cytokine mediators on the regulation of gene expression, and cytochrome P450 metabolism of therapeutic drugs and hypolipidemic agents. More specific uses of the cells of the instant invention include the following.

Drug evaluation and toxicology. Because cultured JB-HEP1 cells maintain xenobiotic function associated with "normal hepatocytes, cells of the instant invention are uniquely qualified to serve as the basis for *in vitro* testing of the response of human hepatocytes to foreign compounds. For example, the intact P450 cytochrome system of JB-HEP1 may be employed to study the xenobiotic metabolism of a host of compounds, including drugs, carcinogens, and hormones. The cells of the present invention are responsive to certain mediators of the acute phase reaction in the

human liver. Accordingly, the cells may be used in developing therapies involved in the treatment of acute phase response(e.g., drugs, cytokines).

<u>Model systems for liver-related diseases.</u>

Cells of the invention provide a means to better understand the biology of liver-associated human-restricted pathogens. Cells of the present invention are particularly useful in the investigation of pathogens such as the Hepatitis A, B, C, D, and E viruses, and *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium vivax*, the latter group of parasites being known to be agents of malaria. In particular, the hepatic cells of the present invention may be provided as an *in vitro* tissue culture, the tissue culture inoculated with a pathogen such as *Plasmodium falciparum* or Hepatitis B virus, and the pathogen grown therein. The pathogen may then be collected from the tissue culture and used for the production of antigens or immunogens useful for the production of diagnostic antibodies and for inducing a protective immune response in a suitable subject.

<u>Hepatocyte-targeted gene transfer.</u> Cells of the invention offer a long-term *in vitro* system for developing and studying gene transfer systems targeted to hepatocytes.

<u>Liver cell transplantation.</u> Cells of the invention are useful for the investigation of long-term storage of isolated hepatocytes by techniques such as cryopreservation. Such stored hepatocytes are useful as repositories of human liver cells, thereby decreasing the dependency on human liver donors for replacement of liver function. Additionally, the cells of the invention may be used to populate a bioartificial liver. Previous attempts to design such bioartificial livers have failed because of the risk of release of cancerous cells into the bloodstream from the tumor-derived cell lines. The cells of the present invention obviate this risk, in that they are not derived from cancer cells but

from normal human liver cells.

Cardiovascular Research The liver has a major role in lipid metabolism, and thus is involved in the development of atherosclerosis and other cardiovascular disorders. Accordingly, the cells of the present invention may be used for the evaluation of hypolipidemic agents designed to be used in the treatment of cardiovascular disease. The cell line is additionally useful in evaluating drugs that lower LDL cholesterol and increase HDL cholesterol. Moreover, the cells of the present invention can be used study other aspects of lipid metabolism, for example, to isolate receptors such as the chylomicron remnant receptor, or the genetic regulation of metabolism.

Verification of traditional research models.

Comparative studies of the human cells of the invention and similar non-human cells are helpful in elucidating interspecies differences which, in turn, are useful for establishing or confirming the value of extrapolating findings obtained from non-human hepatocytes to the analogous human situation. As rodent hepatocytes are often used as a model for human hepatocytes, research comparing rodent hepatocytes with human cells of the instant invention is particularly valuable. Similarly, comparative studies of cells of the invention and human hepatoma cell lines such as HepG2 and He3B should also yield important information regarding the reliability of applied research based on *in vitro* studies of hepatoma cell lines. As hepatoma cells are transformed cells that do not display the "normal" phenotype attributed to the JB-HEP1 cell line, such comparative research should be very useful. The cell line is additionally useful as a model for the study of graft vs. host disease, which disorder relates to the immunology of transplantation of liver and other organs.

The following Examples are provided to further illustrate the present invention, and should not be

construed as limiting thereof. In these Examples, mL means milliters, L means liters, µL means microliters, min means minutes, h means hours, M means molar, mM means millimolar, µM means micromolar, mg means milligrams, µg means micrograms, mm means millimeters, nm means nanometers, µm means micrometers, kB means kilobases, u means units, cpm means counts per minute, mL means milliliter, and kV means kilovolts.

Example 1

Cell isolation and tissue culture

Human fetal hepatic cells were obtained from surgical excess liver tissue from a fetus at 18 weeks gestation, according to the institutional guidelines for the protection of human subjects at the University of North Carolina-Chapel Hill. A small piece of the liver was separated from the connective tissue and the gallbladder. Then the remainder of the tissue, was minced into small pieces with scalpel blades. The minced tissue was passed through folded sterile gauze, and the cells and tissue fragments were washed three times by centrifugation 75 X g for 10 min, in OptiMem (Gibco-BRL Laboratories, Grand Island, New York, USA) containing an antibiotic mixture of 100 µg/mL penicillin G, kanamycin and amphotericin B. Since the tissue was not enzymatically digested, the preparation used for culture was composed of single cells and cell aggregates. The mixture of single cells and cell aggregates (totaling approximately 2 grams of tissue) was resuspended in 50mL of HGM (hepatocyte growth medium) and 4% FBS (Fetal Bovine Serum). 5mL of cell suspension was plated in each of ten, 60mm culture dishes (Corning) and incubated for 24 hours in a humidified atmosphere of 5% CO_2 in air at 37°C. After 24 hours, the cells were rinsed with Hanks' Balanced Salt Solution (HBSS, Gibco-BRL) and 5mL of 20% conditioned serum-free medium (CON/HGM) which was comprised of Ham's-F12/M-199 (1:1; Gibco-BRL) supplemented with MITO+ (per manufacturer's recommendation), ITS (6 µg/mL

insulin, 6μg transferrin and 6ng/mL selenium), bovine pituitary extract (5μg/mL, all from Collaborative Biomedical Products, Bedford, Massachusetts, USA), insulin at 2 mg/L (Sigma) and 50ug/mL of gentamicin sulfate. The supplements were added according to the recommendations of each manufacturer.

Example 2

Preparation of conditioned medium

HepG2 cells (American Type Culture Collection, Rockville, Maryland, USA), were grown in Modified Eagles Medium (Gibco-BRL Laboratories, Logan, UT) for 48 h. The conditioned medium was collected from log phase cultures only. The collected medium was centrifuged to remove cell debris and then filtered through a 0.2um filter (Nalge Company, Rochester, NY). It was then added to the prepared HGM at a concentration of 20%. Conditioned medium was removed permanently from the HGM at passage 50 of the JB-HEP1 cell line, as described below.

Example 3

Transfection of human fetal hepatocytes with the *c-myc* oncogene

The plasmid pRHP1, which was graciously provided by Dr. Russel Kaufman (Duke University), is a 6.5 kB construct containing a portion of human *c-myc* gene(exon 2 and exon 3) driven by an upstream SV-40 promoter cloned into the pBR322 plasmid. The human fetal hepatic cells were plated in ten 60mm culture dishes, and transfection was accomplished by lipofection of the primary cultures on the third day after plating. Cells were transfected with a mixture of 15μl of Lipofectin® (Gibco-BRL, Grand Island, New York, USA) and buffer containing 10 μg of plasmid DNA that had been linearized with *Eco*RI. The DNA and Lipofectin® were mixed according to the manufacturer's instructions, then resuspended in 3 mL of serum-free OptiMem per 60 mm dish. After a 15 min incubation the mixture of linearized plasmid DNA and Lipofectin® was added to six

60mm dishes. Four control dishes were treated in the same manner, except that linearized plasmid DNA was not added to the Lipofectin and medium mixture. The ten dishes were incubated for a period of 24 h under a humidified atmosphere of 5% CO_2 in air at 37°C. After 24 h, medium was replaced with 5mL of 20% conditioned HGM per dish. The culture medium was replaced three times per week.

Example 4

Isolation of proliferative colonies

Transfection of the primary cultures of human fetal hepatic cells was performed three days after attachment. Proliferating cells spread out on the plastic culture plates and differences between the transfected dishes and nontransfected controls could be detected only after about four weeks. Cells in the non-transfected cultures become vacuolated, ceased to replicate, and detached from the culture plates. In contrast, the transfected cells remained attached to the plates and were polyhedral in shape, often in a cordlike appearance reminiscent of hepatic trabeculae with a centrally located spherical nucleus. See **FIG. 1A**. Many cells were binucleate. Nuclei contained one or more nucleoli and scattered heterochromatin clumps. A rim of perinuclear heterochromatin was also prominent, as seen in **FIGS. 1B** and **1C**.

Colonies of tightly packed cells which proliferated and reached a diameter of 1 cm or more within 6-8 weeks were observed in once dish containing the *c-myc* transfected cells. Similar cells were not found in the control dishes. Cells from a plate with established colonies were released from the plate by treatment with a 1:1 mixture of collagenase Type A at 250 μg/mL (Boehringer-Mannheim, Indianapolis, Indiana, USA) and 5000 u/mL of dispase (Collaborative Research). The cell suspension was centrifuged at 75 x g for 5 min, and the pellet was resuspended in unsupplemented growth medium (1:1 mixture of Ham's F12 and M199 and 4% Bovine

Serum) to wash away the collagenase and dispase mixture. The washing procedure was repeated three times. The cells were cultured and expanded in 20% conditioned hepatocyte growth medium (CON/HGM). The ability of the transfected cells to form colonies on a background of senescing normal cells was the only selective procedure used.

All non-transfected control cells senesced after four to eight weeks in culture. Cells transfected with the *c-myc* gene continued to replicate. After 12 weeks in culture, cells were dispersed with 1mL/100mm dish of a 1:1 mixture of 500u/mL dispase and transferred to 100mm dishes. This mixed cell population was propagated through ten passages using only collagenase/dispase dispersion. Starting with passage 11, confluent cell cultures were dissociated with 0.25% trypsin (Gibco) neutralized with 4% FBS in unsupplemented medium, pelleted, resuspended in CON/HGM and subcultured by splitting the confluent cultures 1:4 once a week. The expanded cell culture was designated the JBH1 parental cell line. At passage 11, after trypsinization, 10 dishes of the parental cell line JBH1 had 25-50 single cells per dish remaining attached to the plastic. Rather than discarding these dishes as usual, the dishes were fed normally and the single cells were marked and their growth into colonies was followed. After 6 weeks, 33 colonies were isolated individually because of their hepatocyte-like morphology, based on their apparent trabecular pattern of cell growth. One clone, designated JB-HEP1, was kept in culture, while the rest were cryopreserved. The clonal cell line JB-HEP1 has been propagated for six years, over 250 passages (more than 500 doublings).

Example 5

Chromosomal analysis

The karyotype of the JB-HEP1 human immortalized hepatic cell line was determined based on the examination of over fifty metaphase spreads that

were banded by the trypsin-Giemsa method. Briefly, exponentially growing cells at about 70% confluence were treated with colcemid (0.02µg/mL, Gibco-BRL) for an hour, at 37°C then removed from culture dishes by trypsinization. The cells were suspended in hypotonic (0.075 M) KCl for 20 min at 37°C and fixed 3 times with Carnoy's fixative (3:1 methanol/acetic acid). Fixed cells were dropped onto glass slides to create metaphase spreads. The slides were treated with trypsin (1mg/mL in isotonic buffered saline) for one minute at room temperature and banded with Giemsa stain for five min at room temperature. Metaphase chromosomes were photographed at 100X using 400 speed Kodak t-max film.

The JB-HEP1 hepatocyte cell line showed a prevalence of near tetraploid cells containing a modal number of 69 chromosomes. A typical karyotype for the cells at passage 90 is shown in **FIG. 2**. Although most of the chromosomes appeared to have no detectable abnormalities, some spreads contained marker chromosomes with structural translocations that were not identified for chromosomal origin.

Example 6
Immunohistochemistry

The cells were released by trypsinization and washed in HBSS. 1 x 10^4 cells were cytospun onto glass slides (Fisher, Pittsburgh, Pennsylvania, USA). Antibodies to cytokeratins 8 and 18, and CAM 5.2 (Becton Dickinson, San Jose, California, USA) were used at a 1:10 dilution. Antibodies to cytokeratins 7 and 19 (Dako, Carpinteria, California) were used at 1:40 dilution to document the presence of cytoplasmic keratins. Slides were stained using the Vectastain® Kit, and mouse monoclonal was used for the primary antibody. Common bile duct cells from a normal 18 week human fetal specimen were used as a positive control for cytokeratins 7 and 19, while normal liver parenchyma from an 18 week human fetal specimen were used as a negative control.

The JB-HEP1 cell line was characterized by immunohistochemistry at 90th passage. When the cells grown on slides were reacted with antibodies directed against cytokeratin 8 and 18, an intense positive reaction could be detected by secondary antibody when developed by peroxidase based staining. Staining of the cell line using antibodies directed against cytokeratin 7 was negative, and against cytokeratin 19 was positive. At an earlier passage, these cells were found to be positive for cytokeratins 8 and 18 but negative for both cytokeratins 7 and 19. These results are consistent with hepatic cells of hepatocyte origin and not of bile duct origin. It also indicates that cytokeratin 19 expression reappeared in the immortalized cell line with prolonged culture.

Mature hepatocytes normally express the neutral cytokeratin 8 and the acidic cytokeratin 18. During early fetal development hepatocytes also express keratin 19. *See* P. Stosiek et al., *Liver* **10**, 59-63 (1990). After the 10th week of gestation, keratin 19 is found only in the bile duct cells and it continues to be expressed in bile duct cells in the adults. The fact that these cells ceased to express keratin 19 at an early stage in culture then reexpressed it after further passage may reflect early maturation of hepatocytes in vitro followed later by the loss of this differentiated feature with extensive maintenance in culture. Similarly, these cultured cells expressed the message for alpha-feto protein early during their progagation in vitro but ceased to express it when evaluated at the 90th passage. This is further evidence for the hepatocyte-like nature of these cells and again suggest that these cells undergo maturation during maintenance in culture.

Example 7
Transmission Electron Microscopy

For electron microscopy, cells were plated on plastic culture dishes (Lux-Nunc, Naperville, Illinois,

USA) at low density and allowed to proliferate until they reached confluence. The cultured cells were fixed in situ in 3% gluteraldehyde in serum-free HGM pH 7.2 at 37°C for one hour and post-fixed in 1% osmium tetroxide in 0.1M sodium phosphate buffer, pH7.4 or 1% osmium tetroxide/1.25% potassium ferrocyanide in 0.1M sodium phosphate buffer pH 7.4. Fixed cells were dehydrated through a graded series of ethanol washes, and then embedded in PolyBed 812 (Polysciences Inc., Warrington, Pennsylvania, USA). After curing, the plastic blocks were separated from the culture plates, 70nm ultrathin section were cut, mounted on uncoated copper grids, and double stained with uranyl acetate and lead citrate. The sections were examined at 70Kv with Zeiss Em 10 electron microscope (Carl Zeiss Inc., Oberkochen, Germany) at the magnification indicated.

Transmission electron microscopy showed the cultured JB-HEP1 cells had numerous cytoplasmic organelles, including the Golgi complexes of multiple stacks of five to nine intact cisternae slightly expanded at their end, many round to elongated mitochondria with numerous well preserved cristae, profiles of rough endoplasmic reticulum and free ribosomes, and areas of smooth endoplasmic reticulum that were numerous at the basal side of the cell, with pinocytotic vesicles at the apical side of the cell. See **FIGS. 3A, 3D.** In the area of the cytoplasm rich in smooth endoplasmic reticulum, some of the tubular elements of this organelle contain small, dense spherical particles like newly synthesized very low density serum lipoprotein. The cells had a centrally placed ovoid nucleus with one or several nucleoli. The nuclear structure contained a dense network of granular strands of heterochromatin at the periphery and a double nuclear membrane with distinct nuclear pores. Adjacent cells were joined by desmosomes. Numerous intercellular spaces lined with microvilli resembling bile canaliculi, (**FIG. 3C**) could also been seen between cells this

suggest that the hepatocytes are polarized in culture. Glycogen granules were present and were arranged in clusters to form "rosettes". See **FIG. 3B.** The ultrastructure of the JB-HEP1 cell line demonstrates a phenotype with several features of differentiated human hepatocytes.

Example 8
DNA analysis

High molecular weight DNA was prepared from tissue samples and confluent cell cultures by standard procedures. 10μg of genomic DNA digested with *Eco*RI, was fractionated on a 0.8% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Life Sciences) for hybridization. The membranes were hybridized with a radiolabelled probe prepared with the linearized plasmid used in the transfection, at a specific radioactivity of 10^6 cpm/mL in 3x SSC at 42°C overnight (1xSSC, 0.15M NaCl, 0.015M trisodium citrate). The membranes were washed at (high stringency 0.5x SSC at 55°C), dried and autoradiographed for 1-3 days at-70°C using Kodak XOMAT XAR-5 film.

Example 9
VNTR analysis

The JB-HEP1 cell line was evaluated to distinguish it from several other human specimens and other human cells in culture in the laboratory by evaluating one of their variable genetic markers. The human origin of the JB-HEP1 cell line was verified based on the presence of sequences detected by the variable tandem repeat (VNTR) probe pYNH24 (American Type Culture Collection, Rockville, Maryland, USA) on a Southern blot of *Eco*RI-restricted DNA from the JB-HEP1 cell line. These results also confirmed that the cells at 90th passage contained the same VNTR pattern for this marker as the parental cell line JB-H1. Furthermore, the cultured hepatic cells have a different VNTR pattern than the DNA from several other fetal liver specimens.

Most importantly, the cells have a different VNTR pattern than the HepG2 cell line used to condition medium, and thus they are not a contaminant derived from these cells. Southern blot analysis of *c-myc* of the DNA of the JB-HEP1 cells was done using the *EcoR1* linearized plasmid to detect the transfected copies of *c-myc*. The comparative magnitudes of the label hybridized in the Southern blots was quantitated by densitometry of the autoradiograms. These results indicate that at least a single complete linearized copy of the exogenous *c-myc* gene is integrated in the genome of the JB-HEP1 cell line. See **FIG. 4**.

Example 10
PCR Analysis

The integration of the *c-myc* fragment into the JB-HEP1 was verified by PCR analysis, using a primer homologous to a segment of the upstream SV40 promoter and a primer to the complementary DNA strand homologous to a sequence in the second exon of *c-myc*. The results show that an amplified product of the same size as that produced with the plasmid as template could be generated from JB-HEP1 cells, whereas it is absent from the untransfected control cells. Internal PCR controls verified that PCR amplifications could achieved from the control DNA samples.

Example 11
Total RNA Isolation and Selection of Poly (A)+RNA

Total cellular RNA was isolated from confluent cell cultures using the guanidium isothiocyanate/cesium chloride method as described previously. *See*, J.M. Chirgwin et al., *Biochem*. **18**, 5294-5299 (1979); V. Glisin et al., *Biochem*. **13**, 2633-2638 (1974). Polyadenylated RNA was prepared from CsCl-purified total RNA using oliog-dT cellulose chromatography kits (Pharmacia LKB Biotechnology, Piscataway, New Jersey, USA) according to the manufacturer's instructions.

Example 12
Northern Blot Analysis

Total mRNA for serum protein expression and Poly (A)$^+$ for the *c-myc* expression (20 μg/lane for the former and 5μg/lane for the latter) were fractionated by electrophoresis in 1.0 M formaldehyde, 1% agarose gels for transfer to nylon membranes. After the gel was washed for 1 h in 3 gel volumes of 20x SSC with one wash change after 30 minutes, the RNA was transferred to nylon membrane (Hybond-N®, Amersham Life Sciences) by capillary blotting for 24 h in 20xSSC. RNA was cross-linked to air dried nylon membranes by UV exposure for one min on a UV-Stratalinker-1800 (STRATAGENE). Poly(A)$^+$ selection of total RNA from confluent immortalized cultured hepatocytes was required in order to detect and quantify transcripts of the albumin, fibrinogen and alpha-fetoprotein genes. The positive control for each Northern blot was 20μg of total mRNA or a 5ug aliquot of poly (A)$^+$ RNA isolated from fresh human fetal liver (18 weeks gestation). After suitable autoradiographic exposure, the membranes were stripped of the probe by washing three times in 0.1% SDS at 100°C, and re-hybridized with a new probe as before. See **FIG. 5**.

Example 13
Gene Expression

The presence of specialized ultrastructural characteristics such a bile canaliculi, microvilli and glycogen "rosettes" indicated that the JB-HEP1 cell line was derived from the liver parenchyma, and had retained a differentiated morphology. To determine if this differentiated morphology corresponds with differentiated function, the expression of genes whose expression products are tissue-specific for hepatocytes were examined.

Comparison of the relative levels of gene expression were made by scanning hybridized filters with an Ambis scanning radiation meter. Blots were also

autoradiographed for various times to insure the low and high density bands could be quantitated within the linear range of the X-ray film. Peak integration and background corrections were made by using an LKB gel scanner.

The JB-HEP1 cell line at passage 90 was evaluated for the expression of transcripts for the albumin and alpha-fetoprotein genes as well as gamma-fibrinogen (**FIG. 6**). Albumin was expressed strongly in the JB-HEP1 cells like in the normal fetal hepatocytes and HEPG2 cells. The *c-myc* was expressed at a high level, 5-fold over control hepatocytes from a normal 18 week fetus. However, the expression of the alpha-fetoprotein gene could not be detected at passage 90, whereas this gene had been expressed in the parental cells at passage 30.

In summary, the JB-HEP1 cell line expressed the following: albumin (2.2KB +), gamma-fibrinogen (2.2KB +), and alpha-fetoprotein.

Example 14

Tyrosine aminotransferase assay

Tyrosine aminotransferase (TAT) activity was evaluated because it is a typical example of hormonal regulation of gene function in the liver, and is an indicator of the involvement and integrity of the glucocorticoid receptor in these cells.

For transient TAT enzyme induction experiments, confluent cells were treated by the addition of 10^{-7} dexamethasone to the HGM medium for 24 h. After 24 h the cells were rinsed with cold HBSS, and cell lysis buffer containing 140mM KCl, 125mM KPO_4 (pH 7.6), 1mM EDTA, and 1% NP-40 was added to the cells. Insoluble matter was removed by centrifugation, and protein concentration was determined by BCA protein reagent kit (Pierce Chemical Company, Rockford, Illinois, USA). Tyrosine aminotransferase activities were determined using the method of Granner and Tomkins (*Methods Enzymol*. **17A**, 633-37 (1970)), as modified from

Diamondstone (*Anal. Biochem.* **16**, 395-401 (1966). Enzymatic activities were calculated using the molar extinction coefficient for p-hydroxyphenylpyruvate of 19,900 M^{-1} cm^{-1} and were expressed as nmol of p-hydroxphenylpyruvate formed per minute per microgram of protein.

The levels of TAT enzyme activity were evaluated in the JB-HEP1 cells at the 90th passage both with and without prior induction with 10^{-7} M dexamethasone for 24 hours in culture. Cells from a normal human fetal pancreas, which was used as a negative control, did not have any TAT activity (as expected). Treatment of the JB-HEP1 cell line for a period of 24 h with 10^{-7} M dexamethasone induced the TAT enzyme activity by 11.8-fold as compared to the basal level in uninduced JB-HEP1 cells. These results are presented below in **Table 1.**

Tyrosine amino transferase activity is restricted to hepatocytes but is normally undetectable before birth. *See* O. Greengard, *Biochemical Actions of Hormones Vol. 1*, 53-87 (1970). The cultured cells analyzed above had a low but detectable level of TAT activity. One interpretation of these results is that these cells have undergone post-fetal maturation *in vitro*. The results further suggest that these cells, when treated with dexamethasone, can induce TAT activity and accumulate glycogen, indicating that they remain responsive to some of the gene regulatory processes that operate in normal human hepatocytes. The fact that these same cells accumulate glycogen in response to the treatment with dexamethasone provides further evidence that these cells are hepatocytes because among cells of the liver, glycogen storage capability is unique to hepatocytes. *See, e.g.*, M. Lambiotte et al., *Biochimie* **54**, 1179-87 (1972). In contrast, bile duct epithelial cells do not have the capacity to store glycogen.

Table 1: Expression of TAT enzyme activity in human immortalized fetal hepatocytes

JB/HEP1 +	4.02	11.8 Fold.
JB/HEP1 (+Control)	0.35	base
F18/Pancrease (-Control)	-0.0045	none

The foregoing Examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That Which is Claimed:

1. An immortalized human hepatic cell line, comprising cells characterized by:

(a) an exogenous gene integrated into the cellular genome;

(b) expression of at least one human liver secretory protein; and

(c) morphology consistent with normal hepatocytes.

2. A cell line according to Claim 1, wherein said exogenous gene is the *c-myc* gene.

3. A cell line according to Claim 1, wherein said exogenous gene is a truncated construct of the *c-myc* gene.

4. A cell line according to Claim 1, wherein said cells are derived from normal human fetal cells.

5. A cell line according to Claim 1, wherein said cells are grown in a serum-free medium.

6. A cell line according to Claim 1, wherein said human liver secretory protein is selected from the group consisting of alpha-fetoprotein, gamma-fibrinogen, and albumin.

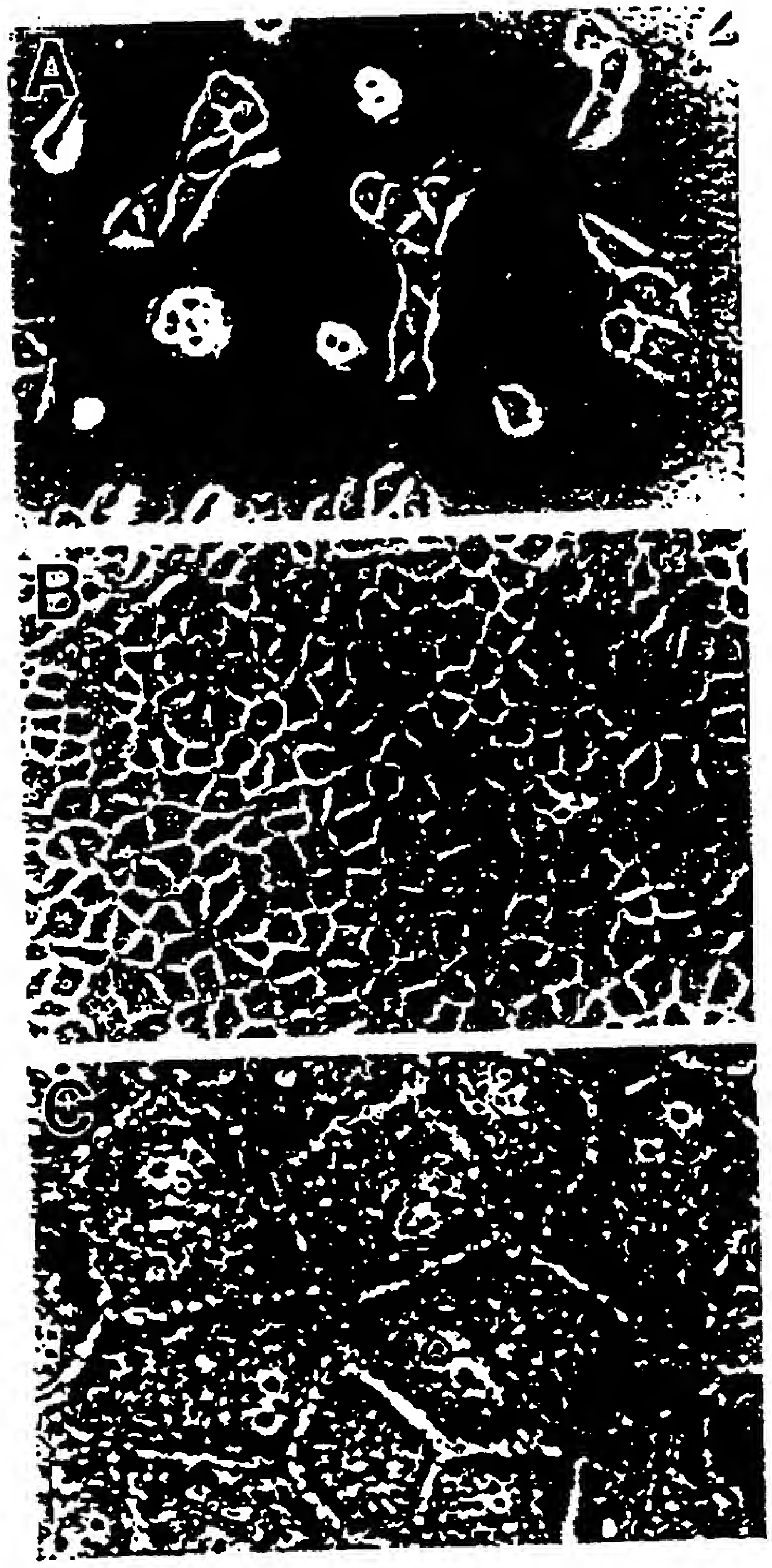

FIG. 1

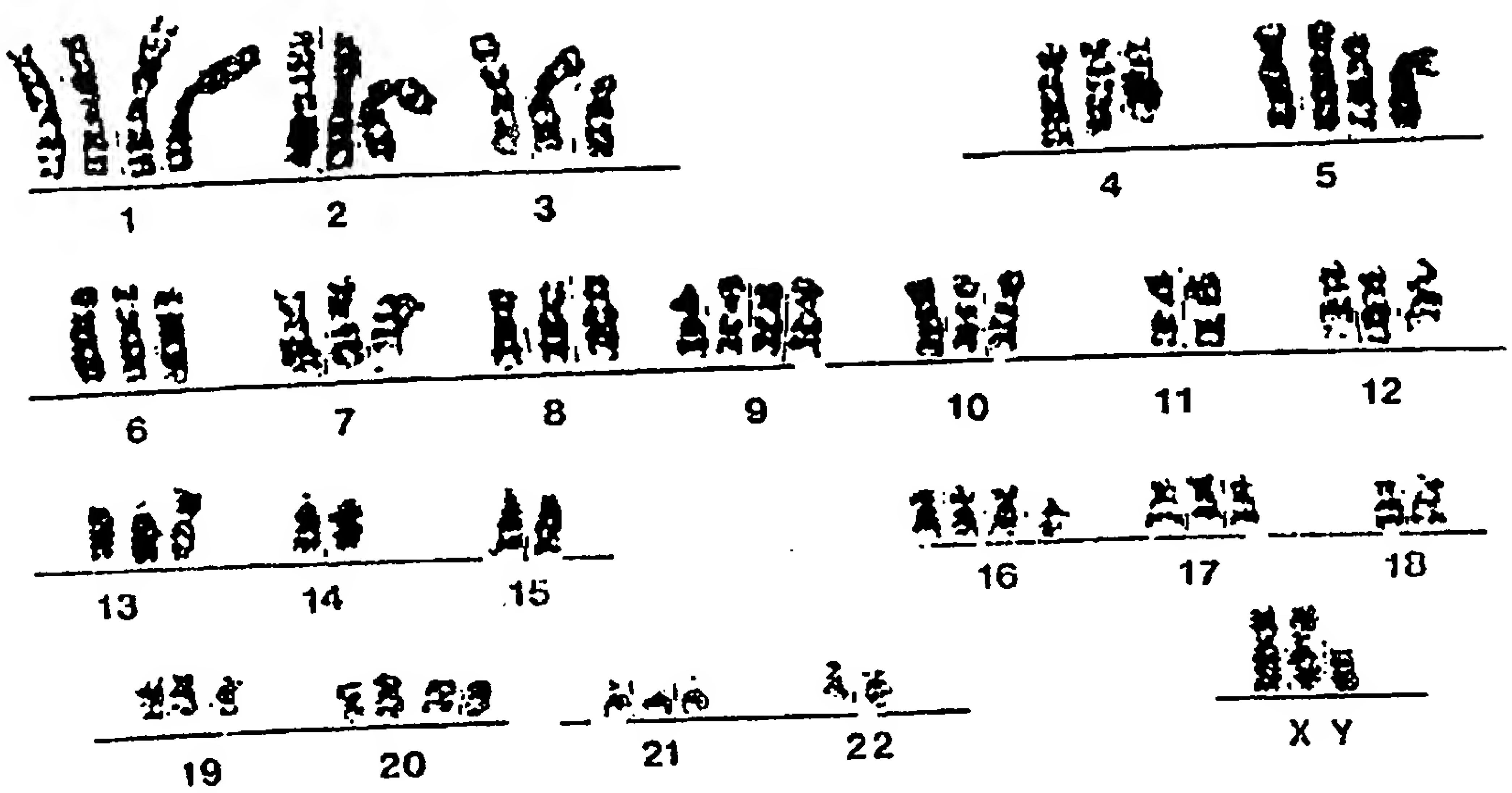

FIG. 2

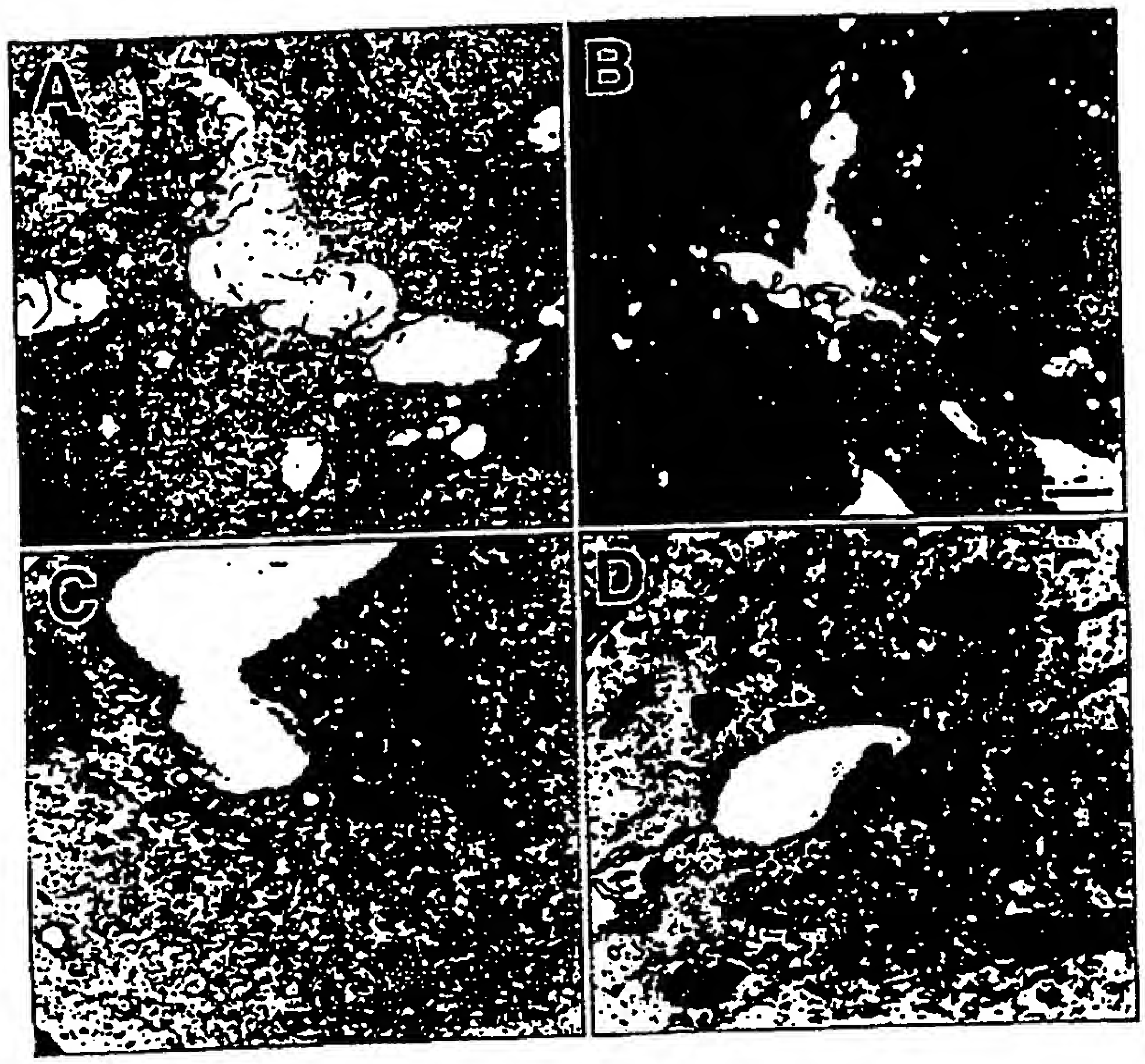

FIG. 3

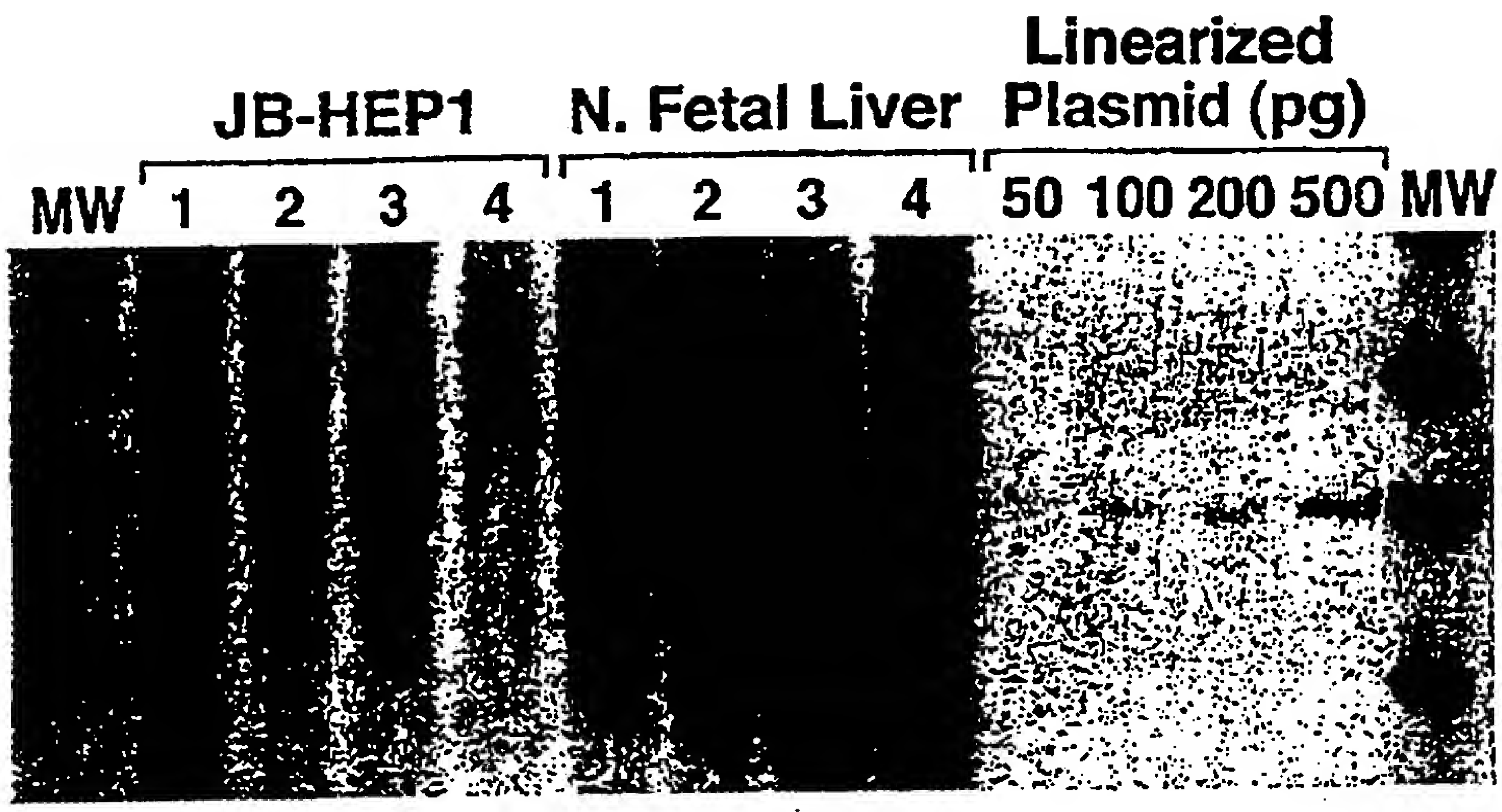
JB-HEP1
N. Fetal Liver
Linearized Plasmid (pg)
MW 1 2 3 4 1 2 3 4 50 100 200 500 MW

FIG. 4

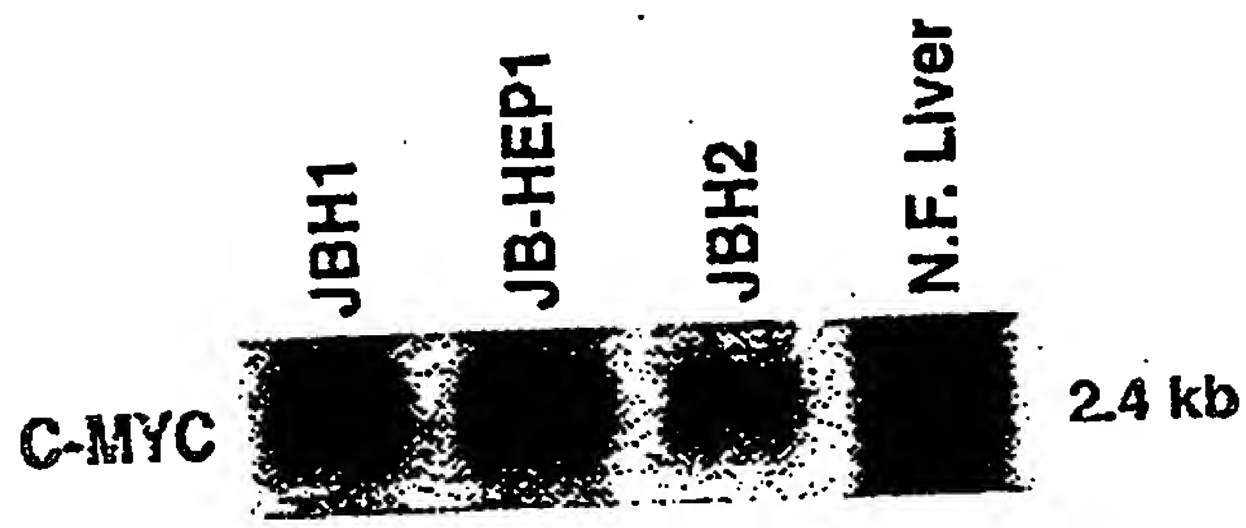

FIG. 5

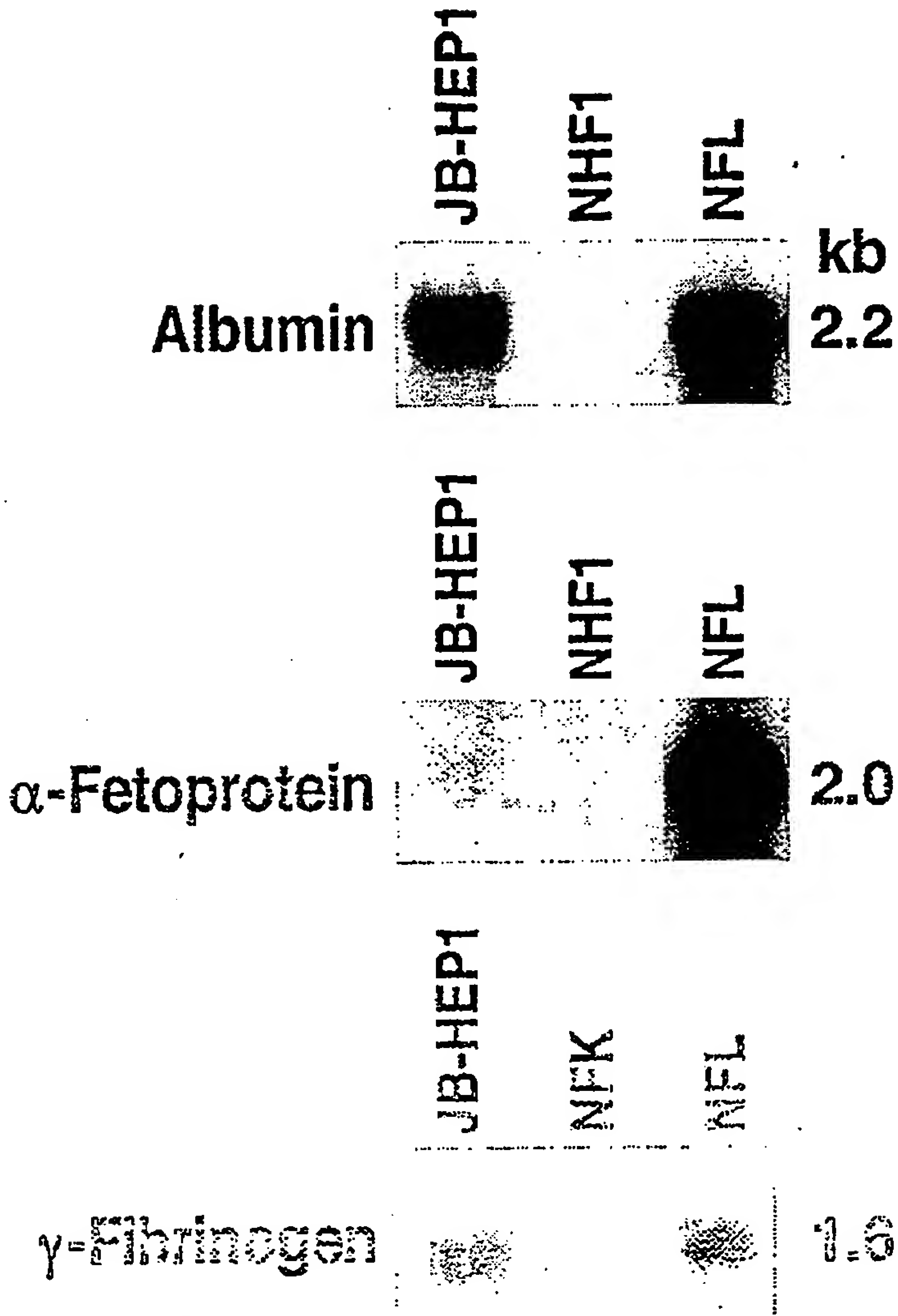
JB-HEP1
NHF1
NFL
kb
Albumin
2.2
JB-HEP1
NHF1
NFL
α-Fetoprotein
2.0
JB-HEP1
NFK
NFL
γ-Fibrinogen
1.6

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14745

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C 12 N 5/08, 5/10
US CL : 435/370
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/370, 363, 366

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	EP 0 527 814 B1 (SOUTHWEST FOUNDATION FOR BIOMEDICAL RESEARCH) 28 December 1994, page 2, lines 39-43; page 3, lines 5-13; page 4, lines 15-34; page 5, lines 5-6; and Table 1, line 46.	1, 2, 4-6 — 2 and 3
Y	ETIEMBLE et al. Fused transcripts of c-myc and a new cellular locus, hcr in a Primary Liver Tumor. Oncogene. January 1989, Vol. 4, No. 1, pages 51-57, especially page 51, Abstract	2 and 3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 OCTOBER 1997	01 DEC 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Brenda Brumback JAB for Telephone No. (703) 308-0196

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG: Medline, BIOTECH, Conf Papers, Euro, WPI
Search terms: immortalized, transformed, liver, hepatitic, hepatocyte(s), c-myc, truncate?, alpha fetoprotein, gamma fibrinogen, albumin